## REMARKS

### Objections to the claims

Claim 36 has been objected to because the marked-up copy and the clean copy of the claim do not match with the amendment filed on July 29, 2002. From the remarks in the Office Action, it appears that the Examiner has worked from the clean copy of the claims. As such, claim 36 has been amended to re-insert the subject matter accidentally deleted from clean copy of claim 36 in the response of July 29, 2002.

Claim 29 has been formally cancelled.

### Rejections under 35 U.S.C.102

The Examiner maintains the rejection of claims 1-12, 14, 18-20, 41-42 and 43 under 35 U.S.C. §102(b) as being anticipated by WO '398. In response to Applicants' arguments of July 29, 2002, the Examiner asserts that WO '398 teaches sequences for targeting to organelle membranes and therefore falls with the scope of the present claims. See specifically, page 6, lines 17-27 of WO '398. Applicants traverse this rejection and withdrawal thereof is respectfully requested.

The invention as defined in claim 1 has been amended to recite that the membrane localization sequence and/or motif is C-terminal to said binding domain. Support for this amendment may be found on pages 19-20, Examples 1 (beginning at page 43) and 2 (beginning at page 53) of the

specification. The presently claimed invention is novel over WO '398 for the following reasons.

As noted above, the present invention is drawn to a fusion protein comprising a membrane anchoring domain which is located C-terminally to the binding domain. As discussed on page 7, lines 23-25 of the specification, membrane localization of such a fusion protein unexpectedly stabilizes the protein and reduces C-terminal degradation in vivo. In addition, transgenic plants expressing such a membrane-anchored fusion protein unexpectedly show an improved resistance against TMV infection. In particular, 16% of infected transgenic plants had no visible disease symptoms on the upper leaves and with 6% of infected transgenic plants had no detectable virus up to 90 days post inoculation. See Example 1, page 43 of the specification. Thus, invention of claim 1 achieves more effective disease resistance in plants than provided by approaches used in the prior art.

The disclosure in WO '398 pertains to soluble antibody-fusion proteins as agents for controlling crop disease caused by pathogens. WO '398 states on page 6, beginning at line 17, that the desired location of the proteins can be achieved using the appropriate targeting sequences. It is well known in the field of cell biology and molecular biology that such "targeting" is mediated by signal sequences which direct the protein in question to the correct location. See for

example, page 294, first full paragraph of the enclosed excerpt of Genes V, Oxford Univ. Press (1994). Such signal sequences must be located at the N-terminus of the protein. See page 296, line 15, left column of Genes V.

According to Genes V, proteins transported to the secretory pathway have significant differences compared to membrane integrated proteins with regard to their respective association with membranes. A protein which is translocated across the ER membrane, i.e. a secreted protein, can be extracted by denaturants that are effective in an aqueous environment. However, the same denaturants are ineffective for extracting proteins that are resident components of the membranes, i.e. membrane integrated proteins. This difference is seen by the fact that translocating proteins move through a channel in the membrane and interact with resident membrane integrated proteins while moving through the membrane structure, with the N-terminal signal sequence directing translocation. The channel in the membrane opens when the nascent polypeptide is transferred from the ribosome to the ER membrane. Resident membrane integrated proteins, on the other hand, interact with the lipid bilayer. Proteins that are integrated into the membrane lipid bilayer have a hydrophobic transmembrane region with external domains on one or both sides of the membrane. The hydrophobic, transmembrane region allows the polypeptide to "float" in the membrane. The majority

of membrane proteins have the N-terminus on the distal side of the membrane with the C-terminal side exposed to the cytosol.

Since the proteins or WO '398 are soluble proteins, the fusion proteins of WO '398 must have an N-terminal signal sequence, i.e. the targeting sequence to direct the fusion protein to the appropriate compartment. See page 6, lines 17-32 of WO '398, which supports this position. However, WO '398 does not disclose a "membrane anchor sequence", which is sequence that must be located C-terminal to the binding domain and which anchors the fusion protein to the desired membrane. Thus, WO '398 fails to disclose each and every feature of the invention of claim 1. As such, the present invention, which requires the membrane anchor sequence to be located C-terminal to the binding domain, is not anticipated by WO '398 and withdrawal of the rejection is respectfully requested.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact MaryAnne Armstrong, PhD (Reg. No. No. 40,069) at the telephone number of the undersigned below.

A marked-up version of the amended sections of the specification and claims is attached hereto.

Applicants request a three (3) month extension of time for responding to the office action. The required fee is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

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0147-0189P

Attachments: Marked-up version showing changes
Excerpt from Genes V (1994)

**MARKED-UP VERSION SHOWING CHANGES**

**IN THE CLAIMS**

Claim 29 has been cancelled.

Claims 1 and 36 have been amended as follows.

1. (Thrice Amended) A fusion protein comprising

(a) at least one binding domain comprising an antibody or binding site thereof that specifically recognizes an epitope of a plant pathogen; and

(b) a membrane localization sequence and/or motif that leads to membrane anchoring,

**wherein said membrane localization sequence and/or motif is C-terminal to said binding domain.**

36. (Twice Amended) A kit comprising the fusion protein of claim 1 **or the pathogenocide of claim 14**.

# GENES V

Benjamin Lewin

OXFORD UNIVERSITY PRESS
Oxford New York Tokyo
1994

[redacted]

## Anchor sequences cause proteins to be retained in membranes

What processes are involved in allowing a (largely) hydrophilic protein to pass through a hydrophobic membrane? The role of an N-terminal signal sequence is essentially to lead the way, as illustrated previously in Figure 11.10. *We assume that once the barrier of the lipid bilayer has been broken by entry of the signal sequence, transfer continues through the membrane until the entire polypeptide has been translocated to the other side. Translocation is initiated by the signal sequence, and is independent of the attached sequences.*

A protein in the process of translocation across the ER membrane can be extracted by denaturants that are effective in an aqueous environment. The same denaturants do not extract proteins that are resident components of the membrane. This suggests the model for translocation illustrated in Figure 11.12. Either the signal sequence inserts into the lipid bilayer at a specific site, or its insertion provides a signal for the creation of such a site, at which proteins of the ER membrane form an aqueous channel through the bilayer. A translocating protein moves through this channel, interacting with the resident proteins rather than with the lipid bilayer.

Such channels can be detected by their ability to allow the passage of ions (measured as a localized change in electrical conductance). The channel

**Figure 11.12**

Only the signal sequence need interact directly with the hydrophobic environment of the lipid bilayer. The remaining sequences of a protein translocating through the membrane may move through an aqueous tunnel created by resident ER membrane proteins.

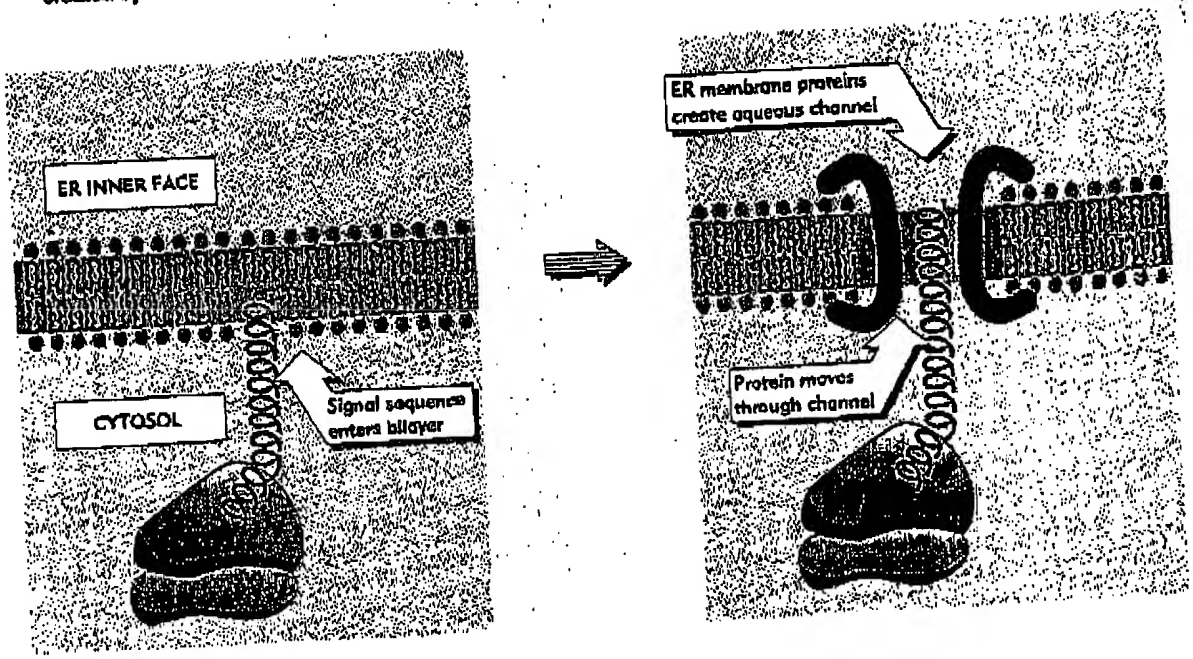
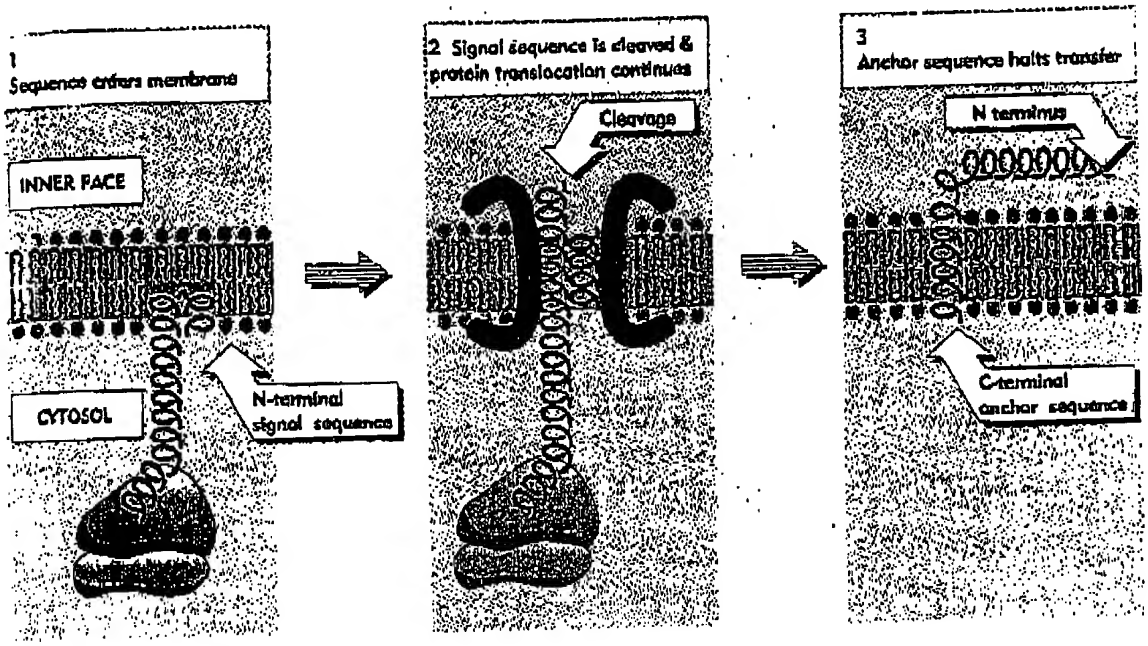


**Figure 11.13**

Proteins that reside in membranes enter by the same route as secreted proteins, but transfer is halted when an anchor sequence passes into the membrane. If the anchor is at the C-terminus, the bulk of the protein passes through the membrane and is exposed on the far surface.




appears to open when a nascent polypeptide is transferred from a ribosome to the ER membrane. The translocating protein fills the channel completely, because ions cannot pass through during translocation. But if the protein is released by treatment with puromycin, then the channel becomes freely permeable. If the ribosomes are removed from the membrane, the channel closes, suggesting that the open state requires the presence of the ribosome.

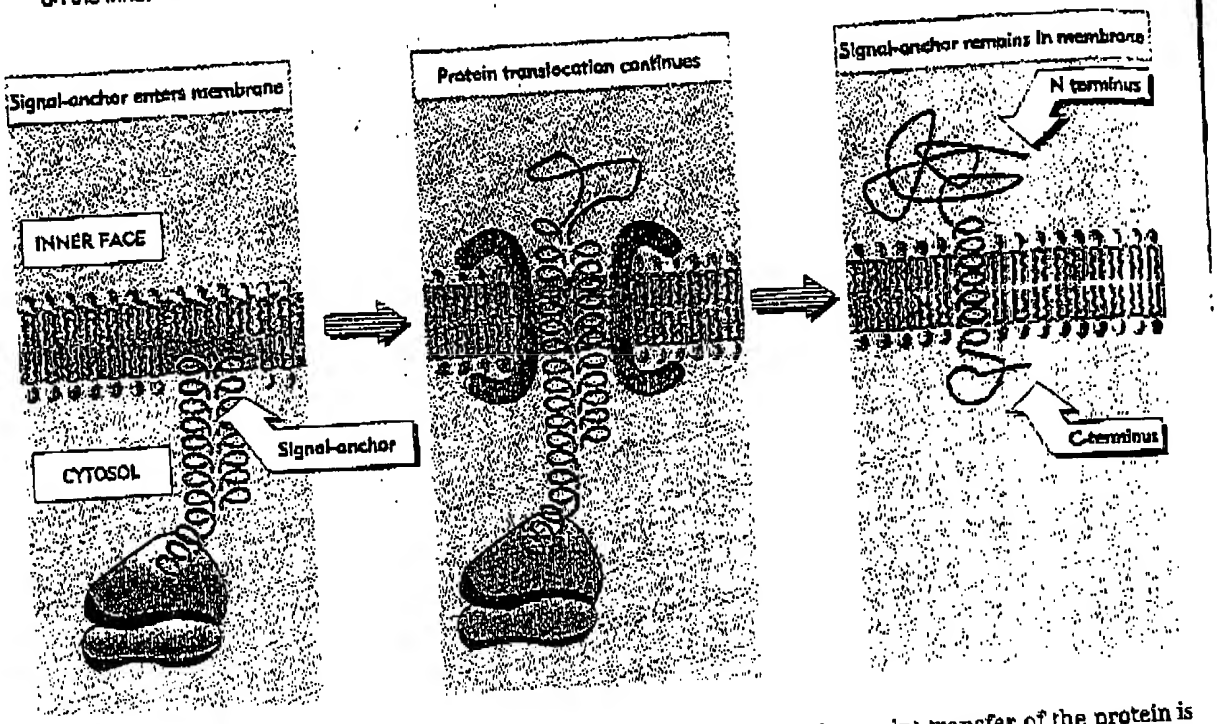
How are proteins that are secreted *through* the ER membrane distinguished from those that reside *within* it? Integral membrane proteins have hydrophobic regions that actually are located within the lipid bilayer, while external domains are located on one or the other side of the membrane. A hydrophobic region provides a transmembrane domain that enables the polypeptide to float in the membrane. It is organized as an α-helix, 21–26 residues long, which forms a coil that can span the lipid bilayer (see Figure 2.6).

The signals that are responsible for insertion of proteins in membranes determine the orientation of the protein. Proteins that have the N-terminus on the far side of the membrane, while the C-terminus remains exposed to the cytosol where it was synthesized, are called type I membrane proteins. They comprise the majority of membrane proteins. A smaller number of type II integral membrane proteins have the opposite orientation; they display an N-terminal domain on the cytosolic side and expose the C-terminal domain on the extracellular side.

The pathway by which proteins of either type I or type II are inserted into the membrane follows the same initial route as that of secretory proteins, relying on a signal sequence that functions co-translationally. But proteins that are to remain within the membrane possess a second,

**Figure 11.14**

A combined signal-anchor sequence causes a protein to reverse its orientation, so that the N-terminus remains on the inner face and the C-terminus is exposed on the outer face of the membrane.




stop-transfer signal. This takes the form of a cluster of hydrophobic amino acids adjacent to some ionic residues. The cluster serves as an anchor that latches onto the membrane and stops the protein from passing right through. We do not know how it functions.

Membrane insertion starts for both types of protein by the insertion of a signal sequence in the form of a hairpin loop, in which the N-terminus remains on the cytoplasmic side. Two features determine the position and orientation of a protein in the membrane: whether the signal sequence is cleaved; and the location of the anchor sequence.

The insertion of type I proteins is illustrated in Figure 11.15. The signal sequence is always N-terminal. When it is attacked by the peptidase on the lumenal side of the membrane, the cleavage releases a new N-terminus that can continue into the lumen. The location of the anchor signal determines at what point transfer of the protein is halted. When the anchor sequence takes root in the membrane, domains on the N-terminal side will be located in the lumen, while domains on the C-terminal side are located facing the cytosol.

A common location for a stop-transfer sequence of this type is at the C-terminus. As shown in the figure, transfer is halted only as the last sequences of the protein enter the membrane. This type of arrangement is responsible for the location in the membrane of many proteins, including cell surface proteins. Most of the protein sequence is exposed on the lumenal side of the membrane, and it is a sequence at or close to the C-terminus that anchors the protein in the membrane.

Type II proteins do not have a cleavable leader sequence at the N-terminus. Instead the signal sequence is combined with an anchor sequence. We imagine that the general pathway for the in-

tegration of type I proteins into the membrane involves the steps illustrated in Figure 11.14. The signal sequence enters the membrane, but the joint signal–anchor sequence does not pass through. Instead it sticks in the lipid phase of the membrane, while the rest of the growing polypeptide continues to loop into the endoplasmic reticulum.

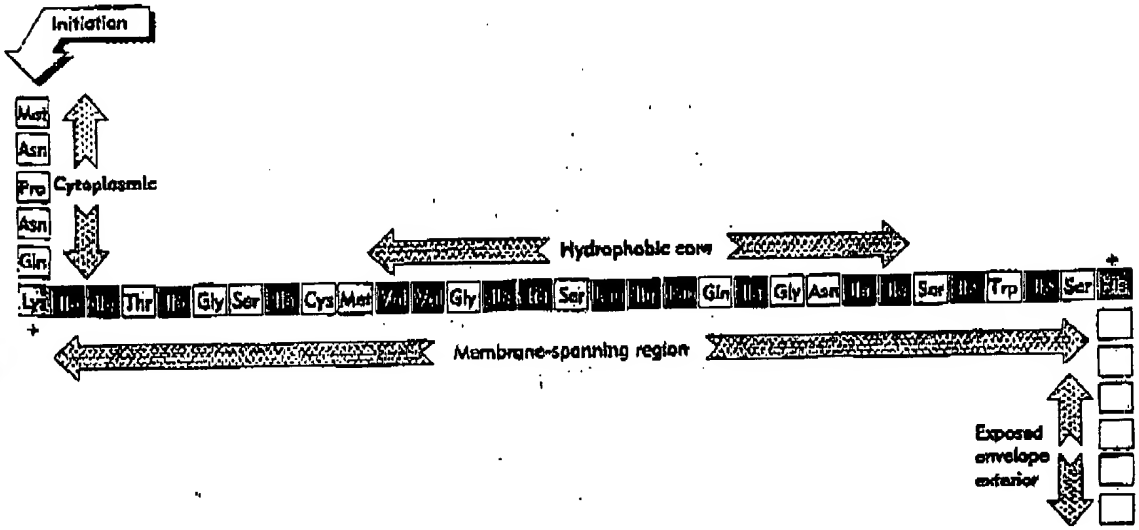
The signal–anchor sequence is usually internal, and its location determines which parts of the protein remain in the cytosol and which are extracellular. Essentially all the N-terminal sequences that precede the signal–anchor are exposed to the cytosol. Usually this cytosolic tail is short, ~6–30 amino acids. In effect the N-terminus remains constrained while the rest of the protein passes through the membrane. This reverses the orientation of the protein with regard to the membrane. (We discuss various types oforientation for membrane proteins in more detail in Chapter 12.)

Anchor sequences are less well defined than signal sequences, but fit the general criteria for transmembrane domains. A surprising property of anchor sequences is that they can function as signal sequences when engineered into a different location. When placed into a protein lacking other signals, such a sequence may sponsor membrane translocation. One possible explanation for these results is that the signal sequence and anchor sequence interact with some common component of the apparatus for translocation. Binding of the signal sequence initiates translocation, but the appearance of the anchor sequence displaces the signal sequence and halts transfer. In the absence of any prior signal sequence, an anchor sequence therefore might be able to initiate translocation.

The combined signal–anchor sequence of type II proteins resembles a cleavable signal sequence. Figure 11.15 gives an example. Like cleavable leader sequences, the amino acid composition is more important than the actual sequence. The regions at the extremities of the signal–anchor carry positive charges; the central region is uncharged and resembles a hydrophobic core of a cleavable leader. Mutations to introduce charged amino acids

**Figure 11.15**

The signal-anchor of influenza neuraminidase is located close to the N-terminus and has a hydrophobic core.



in the core region prevent membrane insertion; mutations on either side prevent the anchor from working, so the protein is secreted or located in an incorrect compartment.

The distribution of charges around the anchor sequence has an important effect on the orientation of the protein. More positive charges are usually found on the cytoplasmic side (N-terminal side in type II proteins). If the positive charges are removed by mutation, the orientation of the protein can be reversed. The effect of charges on orientation is summarized by the 'positive inside' rule, which says that the side of the anchor with the most positive charges will be located in the cytoplasm. The positive charges in effect prove a hook that latches on to the cytoplasmic side of the membrane, controlling the direction in which the hydrophobic region is inserted, and thus determining the orientation of the protein.

The process of insertion into a membrane has been characterized for both type I proteins (Figure 11.15) and type II proteins (Figure 11.14), in which there is a single transmembrane domain. But some membrane proteins have a more complex organization in which there are multiple transmembrane domains, separated from one another by sequences that loop out into the extracellular space on one side or the cytosol on the other (see Figure 12.4).

An interesting question is how an integral membrane protein initially moves into the lipid bilayer. As it passes through the channel illustrated in Figure 11.12, it must reach a point at which the channel is occupied by the hydrophobic transmembrane region. Then this region must be transferred laterally from the channel to the lipid bilayer. This could be accomplished by a dissociation of the channel into its components or perhaps by some rearrangement that exposes the hydrophobic region to the surrounding lipids.

How is a protein with multiple membrane-spanning regions inserted into a membrane? Much less is known about this process, but we assume that it relies on sequences that provide signal and/or anchor capabilities. One model is to suppose that there is an alternating series of signal and anchor sequences. Translocation is initiated at the first signal sequence and continues until stopped by the first anchor. Then it is reinitiated by a subsequent signal sequence, until stopped by the next anchor. Another possibility is that, once one transmembrane domain has been inserted, subsequent transmembrane domains become localized in the membrane because they interact with a domain already located there.

## Bacterial proteins are transported by both co-translational and post-translational mechanisms

The secretion of proteins from bacteria relies on mechanisms very similar to those characterized for eukaryotic cells. Transport from the bacterial cytoplasm passes through the inner membrane into the periplasmic space and then (sometimes) through the outer membrane into the environment. Co-translational transfer is common in *E. coli*, but is not universal. Some proteins are secreted both co-translationally and post-translationally. The relative kinetics of translation versus secretion through the membrane could determine the balance.

Exported bacterial proteins have N-terminal leader sequences, with a hydrophilic N-terminus and an adjacent hydrophobic core. Mutations in N-terminal leaders prevent secretion; they are suppressed by mutations in other genes, which are thus defined as components of the protein export apparatus. Several genes given the general description *sec* are implicated in coding for components of the secretory apparatus by the occurrence of mutations that block